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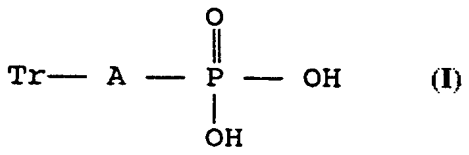
(54) Title: TRIAZOLE PHOSPHONATE PESTICIDES

(57) Abstract



(I)

Compounds of formula (I), where Tr is optionally substituted 1,2,4-triazol-1-yl or 1.2.4-triazol-3-yl; A is a) Q, where Q is an optionally substituted chain containing 3 to 6 atoms, and in which (i) when the chain is of 3 atoms, at least one of the chain atoms is a hetero atom, (ii) when the chain comprises 4 carbon atoms, then the second carbon from the triazole is not substituted by optionally substituted hydroxy, and (iii) when the chain is of 3 to 5 atoms, the phospho group is not attached directly to an oxygen atom; or b) an optionally substituted three membered carbon chain or ring, in which two optional substituents can together with the chain atoms form a carbocyclic ring; and in which when A is a chain, various proviso apply, can be used for combating fungi. The compounds have been shown to be inhibitors of imidazole glycerol phosphate dehydratase. Many of the compounds are novel and these also have other pesticidal activity.

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Title: Triazole phosphonate pesticides

Field of the invention

This invention relates to pesticides based on triazole linked to a phosphonate group.

Prior Art

In GB 2158071 there are disclosed, inter alia, [3-(1,2,4-triazolyl)-2-hydroxypropyl]phosphonic acid derivatives having fungicidal activity. In WO 92/19629, there are disclosed, inter alia, other (triazolylalkyl)phosphonic acid derivatives and especially [3-(1,2,4-triazolyl)-3-hydroxypropyl]- and [3-(1,2,4-triazolylcarbonyl)ethyl]-phosphonic acid derivatives which have herbicidal activity, but are also claimed to have fungicidal activity. Similar compounds with herbicidal activity are disclosed in GB 2271113.

In EP 528760 there are disclosed [3-(1,2,4-triazolyl)-3-hydroxypropyl]phosphonic acid derivatives (excluding those claimed in WO 92/19629), for which herbicidal activity only was exemplified, but are also claimed to have fungicidal activity.

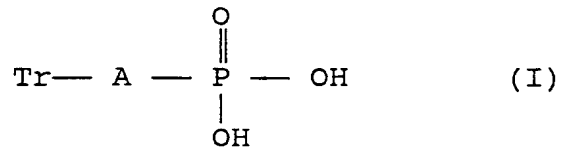
In WO 93/15610 there are disclosed as herbicides, compounds based on triazole linked to a phosphonate group by a optionally substituted chain or ring of three carbon atoms. Similar herbicidal compounds are disclosed in EP 78613.

We have found that some of these compounds previously disclosed only as having herbicidal activity also have fungicidal activity. We have also discovered that certain novel derivatives based on triazole linked to a phosphonate group have pesticidal and especially

fungicidal and/or herbicidal activity.

Disclosure of the invention

According to the invention there is provided the use for combating fungi, of a compound of formula I



(I)

where

Tr is optionally substituted 1,2,4-triazol-1-yl or 1,2,4-triazol-3-yl;

A is

a) Q, where Q is an optionally substituted chain containing 3 to 6 atoms, and in which

(i) when the chain is of 3 atoms, at least one of the chain atoms is a hetero atom,

(ii) when the chain comprises 4 carbon atoms, then the second carbon from the triazole is not substituted by optionally substituted hydroxy, and

(iii) when the chain is of 3 to 5 atoms, the phospho group is not attached directly to an oxygen atom; or

b) an optionally substituted three membered carbon chain or ring, in which two optional substituents can together with the chain atoms form a carbocyclic ring; and in which when A is a chain, then

(i) when the carbon adjacent Tr is substituted by oxo or optionally substituted hydroxy, at least one of the other chain carbons is substituted, and/or two optional substituents can together with the chain atoms form a carbocyclic ring;

(ii) when Tr is unsubstituted 1,2,4-triazol-1-yl and the carbon adjacent Tr is unsubstituted or substituted by alkyl, aryl or haloaryl, the middle chain carbon is not substituted by hydroxy,

together with esters, salts and complexes with metal salts thereof.

Preferably, Q comprises 4 or 5 atoms and especially 5 atoms.

Generally, the compound is present as the free acid or salt thereof, though in certain cases metabolisable esters, e.g. acyloxyalkyl or phenyl may be advantageous.

Many of the compounds of formula I are novel and the invention thus includes compounds of general formula I in which A is as defined under (a), as well as acyloxyalkyl esters of compounds where A is as defined under (b).

In Q, the preferred hetero atoms are nitrogen, oxygen, phosphorus or sulfur. The nitrogen, sulfur and phosphorus may be oxidised.

Optional substituents on carbon atoms forming A include an aliphatic hydrocarbon radical, which may be unsaturated, amino, hydrazono, hydroxy, mercapto or hydroximino, each of which is optionally substituted, acyl, carboxy (and salt and ester derived forms thereof), halogen, azido, nitro, cyano, oxo, aryl or heterocyclyl. Optional substituents on nitrogen atoms forming A, include acyl or aryl, or alkyl, amino or hydroxy, each of which is optionally substituted.

Optional substituents on the triazolyl, include alkyl, e.g methyl, acyl and other protecting groups, especially trityl, acyloxymethyl and dimethylsulfamoyl.

Aliphatic hydrocarbon radicals are generally of up 10 carbon atoms and can be cyclic or acyclic. Acyclic groups may branched or straight chained. Substituents, when

present on any aliphatic hydrocarbon radical group, halogen, cyano, alkoxy (e.g. of 1 to 4 carbon atoms, and which may be optionally substituted, e.g. by halo), hydroxy, alkylthio, nitro, optionally substituted amino, carboxy (and salt and ester derivatives thereof), acyl, acyloxy or aryl. Cyclic aliphatic groups are generally of 3 to 8 carbon atoms.

Aryl groups are usually phenyl, optionally substituted, e.g. by halogen, cyano, nitro, optionally substituted alkyl, alkylthio or alkoxy, aryl, aryloxy, nitro, optionally substituted amino, optionally substituted carbamoyl, carboxy, optionally substituted alkoxycarbonyl, In some cases two substituents, together with the phenyl to which they are attached, can form a fused ring which itself can be optionally substituted as for phenyl.

The term heterocyclyl includes aromatic and non-aromatic rings which usually contain 5 to 7 ring atoms and including up to three hetero atoms usually selected from nitrogen, oxygen and sulfur. Examples of such groups include thienyl, furyl, pyridyl, pyrimidinyl, pyrazolyl, thiazolyl, thiazolinyl, thiazolone, oxazolyl, benzimidazolyl, tetrazolyl, benzoxazolyl, thiadiazolyl, dioxolanyl, imidazopyridinyl, 1,3-benzoxazinyl, 1,3-benzothiazinyl, oxazolopyridinyl, triazolyl, triazinyl, imidazolyl, morpholino, benzofuranyl, pyrazolinyl, quinolinyl, quinazolinyl, dihydroquinazolinyl or benzothiazolyl. The heterocyclyl group may be substituted, e.g. as described for phenyl.

Amino and hydrazono groups may be substituted, e.g. by one or two optionally substituted alkyl, aryl or acyl groups or two substituents can form a ring, e.g. a morpholino or piperidino ring.

Hydroxy, mercapto or hydroximino groups may be substituted, e.g. by one or two optionally substituted alkyl, aryl or acyl groups.

The term acyl includes the residue of sulfur and phosphorus-containing acids as well as carboxylic acids. Examples of acyl groups are thus $-COR^5$, $-COOR^5$, $-CXNR^5R^6$, $-CON(R^5)OR^6$, $-COONR^5R^6$, $-CON(R^5)NR^6R^7$, $-COSR^5$, $-CSSR^5$, $-S(O)_pR^5$, $-S(O)_2OR^5$, $-S(O)_pNR^5R^6$, $-P(=X)(OR^5)(OR^6)$, $-CO-COOR^5$, where R^5, R^6 and R^7 which may be the same or different, are hydrogen, an aliphatic hydrocarbon radical, which may be unsaturated, aryl or heterocyclyl or R^6 and R^7 together with the atom(s) to which they are attached can form a ring. p is 1 or 2 and X is O or S.

It will be evident to those skilled in the art that in certain compounds of formula I, there exists the possibility of tautomeric forms of these molecules. These tautomer and any mixtures thereof form part of the invention. It will also be appreciated that some compounds may comprise a group with a chiral carbon or sulfur atom resulting in the possibility of enantiomers and the invention includes individual enantiomers as well as mixtures of these. The invention also includes individual geometric isomers where these may exist.

Salts of compounds of the invention are usually those of agriculturally acceptable metal cations or of organic bases, especially tertiary amines.

Complexes of compounds of the invention are usually formed from a salt of formula MAn_2, in which M is a divalent metal cation, e.g. copper, manganese, cobalt, nickel, iron or zinc and An is an anion, e.g. chloride, nitrate or sulfate.

The compounds of the invention are particularly suitable for combating phytopathogenic fungi, such as mildews and particularly barley powdery mildew (*Erysiphe graminis*) and vine downy mildew (*Plasmopara viticola*), rice blast (*Pyricularia oryzae*), late tomato or potato blight (*Phytophthora infestans*), apple scab (*Venturia inaequalis*) and glume blotch (*Leptosphaeria nodorum*). Other fungi against which the compounds may be active include other powdery mildews, other rusts, and general pathogens of Deuteromycete, Ascomycete, Phycomycete and Basidomycete origin.

The compounds may also have activity against wood damaging fungi and also human pathogenic fungi, such as *Candida albicans*.

The novel compounds of the invention as well as, or instead of, fungicidal activity may have other pesticidal activity and especially herbicidal activity.

We have also discovered that the fungicidal and herbicidal activity shown by certain compounds of formula I derives from their inhibition of imidazole glycerol phosphate dehydratase (IGPD), which is an enzyme in the histidine biosynthetic pathway. This herbicidal mode of action has been proposed by several authors (see for example G. M. Kishore and D.M. Shah, Annu. Rev. Biochem. 1988, 57, 627, and references therein). We are unaware of this mode of action having been disclosed for fungicides. Indeed it is surprising that this enzyme is a fungicidal target since one might have assumed that the fungus would obtain histidine from the host plant.

The physiological and biochemical responses caused by certain compounds of formula I are not characteristic of known modes of action. For example treatment of

Leptosphaeria nodorum with [3-hydroxy-3-(1,2,4-triazol-3-yl)cyclohexyl]-phosphonic acid, (compound 2.002 in EP 528760) inhibits mycelial growth on minimal media. Supplementing the media with L-histidine overcame growth inhibition by this compound, but supplementing with other amino acids such as L-lysine and L-arginine had no effect. This reversal of fungicide activity by L-histidine was also demonstrated on wheat plants infected with *Leptosphaeria nodorum*. The fungicide activity shown by this compound was unaffected when the compound was applied to plants with D-histidine, but the fungicide activity was lost when applied with L-histidine. These observations are consistent with the primary target of such compounds being an enzyme in the histidine biosynthetic pathway. The enzyme, IGPD, is in fact extremely sensitive to certain compounds of formula I in *in vitro* tests. These results establish IGPD inhibition as the mode of action for these compounds. This is a novel fungicidal mode of action and its discovery opens up the opportunity of identifying novel chemical compounds which inhibit the same enzyme target.

Although the IGPD assay will identify the intrinsic fungicidal activity of a compound, it is subsequently necessary to use conventional tests to confirm the *in vivo* fungicidal activity.

We have found a strong correlation between activity of a compound in the IGPD assay and fungicidal activity.

Thus the invention also provides a method for identifying potential fungicides which comprised testing a candidate compound in an IGPD inhibition assay and also includes fungicides identified using this test.

The invention still further provides the use as a

fungicide of a compound which is an IGPD inhibitor, with the proviso that the compound is not a general enzyme inhibitor and is not a compound previously known to have fungicidal activity.

In this use, the IGPD inhibitor is one which produces at least a 20%, and preferably at least a 50%, reduction in IGPD activity when tested against a fungal enzyme preparation at 100 μM or less. One suitable enzyme preparation is that derived from *Saccharomyces cerevisiae*.

The compounds of the invention have activity against a wide range of pathogens of Deuteromycete, Ascomycete, Phycomycete and Basidiomycete origin, and especially against fungal diseases of plants, e.g. mildews and particularly cereal powdery mildew (*Erysiphe graminis*) and vine downy mildew (*Plasmopara viticola*), rice blast (*Pyricularia oryzae*) and glume blotch (*Leptosphaeria nodorum*).

Some compounds also have herbicidal activity, against a wide range of undesirable weeds such as those disclosed in Test Example 2.

The compounds of the invention are generally formulated in conventional compositions used for pesticides. These compositions can contain one or more additional pesticides, for example compounds known to possess herbicidal, fungicidal, insecticidal, acaricidal or nematicidal properties.

The diluent or carrier in the composition of the invention can be a solid or a liquid optionally in association with a surface-active agent, for example a dispersing agent, emulsifying agent or wetting agent. Suitable surface-active agents include anionic compounds such as a

carboxylate, for example a metal carboxylate of a long chain fatty acid; an N-acylsarcosinate; mono- or di-esters of phosphoric acid with fatty alcohol ethoxylates or salts of such esters; fatty alcohol sulfates such as sodium dodecyl sulfate, sodium octadecyl sulfate or sodium cetyl sulfate; ethoxylated fatty alcohol sulfates; ethoxylated alkylphenol sulfates; lignin sulfonates; petroleum sulfonates; alkyl-aryl sulfonates such as alkyl-benzene sulfonates or lower alkylnaphthalene sulfonates, e.g. butyl-naphthalene sulfonate; salts of sulfonated naphthalene-formaldehyde condensates; salts of sulfonated phenol-formaldehyde condensates; or more complex sulfonates such as the amide sulfonates, e.g. the sulfonated condensation product of oleic acid and N-methyl taurine or the dialkyl sulfosuccinates, e.g. the sodium sulfonate of dioctyl succinate. Nonionic agents include condensation products of fatty acid esters, fatty alcohols, fatty acid amides or fatty-alkyl- or alkenyl-substituted phenols with ethylene oxide, fatty esters of polyhydric alcohol ethers, e.g. sorbitan fatty acid esters, condensation products of such esters with ethylene oxide, e.g. polyoxyethylene sorbitan fatty acid esters, block copolymers of ethylene oxide and propylene oxide, acetylenic glycols such as 2,4,7,9-tetramethyl-5-decyne-4,7-diol, or ethoxylated acetylenic glycols. Examples of a cationic surface-active agent include, for instance, an aliphatic mono-, di-, or polyamine as an acetate, naphthenate or oleate; an oxygen-containing amine such as an amine oxide or polyoxyethylene alkylamine; an amide-linked amine prepared by the condensation of a carboxylic acid with a di- or polyamine; or a quaternary ammonium salt.

The compositions of the invention can take any form known in the art for the formulation of agrochemicals, for example, a solution, a dispersion, an aqueous emulsion, a

dusting powder, a seed dressing, a fumigant, a smoke, a dispersible powder, an emulsifiable concentrate or granules. Moreover it can be in a suitable form for direct application or as a concentrate or primary composition which requires dilution with a suitable quantity of water or other diluent before application.

As a dispersion, the composition comprises a compound of the invention dispersed in a liquid medium, preferably water. It is often convenient to supply the consumer with a primary composition which can be diluted with water to form a dispersion having the desired concentration. The primary composition can be provided in any one of the following forms. It can be a dispersible solution which comprises a compound of the invention dissolved in a water-miscible solvent with the addition of a dispersing agent. A further alternative comprises a compound of the invention in the form of a finely ground powder in association with a dispersing agent and intimately mixed with water to give a paste or cream which can if desired be added to an emulsion of oil in water to give a dispersion of active ingredient in an aqueous oil emulsion.

An emulsifiable concentrate comprises a compound of the invention dissolved in a water-immiscible solvent together with an emulsifying agent and which is formed into an emulsion on mixing with water.

A dusting powder comprises a compound of the invention intimately mixed with a solid pulverulent diluent, for example, kaolin.

A granular solid comprises a compound of the invention associated with similar diluents to those which may be employed in dusting powders, but the mixture is granulated

by known methods. Alternatively it comprises the active ingredient adsorbed or absorbed on a pre-granular diluent, for example, Fuller's earth, attapulgite or limestone grit.

A wettable powder usually comprises the active ingredient in admixture with a suitable surfactant and an inert powder diluent such as china clay.

Another suitable concentrate, particularly when the product is a solid, is a flowable suspension concentrate which is formed by grinding the compound with water, a wetting agent and a suspending agent.

The concentration of the active ingredient in the composition of the present invention, as applied to plants is preferably within the range of 0.001 to 3.0 per cent by weight, especially 0.01 to 0.1 per cent by weight. In a primary composition the amount of active ingredient can vary widely and can be, for example, from 5 to 95 per cent by weight of the composition.

In the method of the invention the compound is generally applied to seeds, plants or their habitat. Thus the compound can be applied directly to the soil before, at or after drilling so that the presence of active compound in the soil can control the growth of the pest (e.g fungus or weed). When the soil is treated directly the active compound can be applied in any manner which allows it to be intimately mixed with the soil such as by spraying, by broadcasting a solid form of granules, or by applying the active ingredient at the same time as drilling by inserting it in the same drill as the seeds. A suitable application rate is within the range of from 0.05 to 20 kg per hectare, more preferably from 0.1 to 10 kg per hectare.

Alternatively the active compound can be applied directly to the plant by, for example, spraying or dusting either at the time when the pest (e.g fungus or weed) has begun to appear or before the appearance of the pest as a protective measure. In both such cases the preferred mode of application is by foliar spraying. When the active compound is applied directly to the plant a suitable rate of application is from 0.001 to 5 kg per hectare, preferably from 0.01 to 0.5 kg per hectare.

The novel compounds of the invention may be prepared using known methodology, in a variety of ways, for example: compounds which are acids may be obtained by de-esterifying, in known manner, the corresponding esters. The esters can be obtained in known manner. Salts may be obtained from acids or esters in conventional manner.

Generally compounds are preferred in which the 1,2,4-triazolyl moiety is unsubstituted. However in certain processes it is necessary for the triazole to be protected and the protected group can then be removed at a final or penultimate synthesis stage.

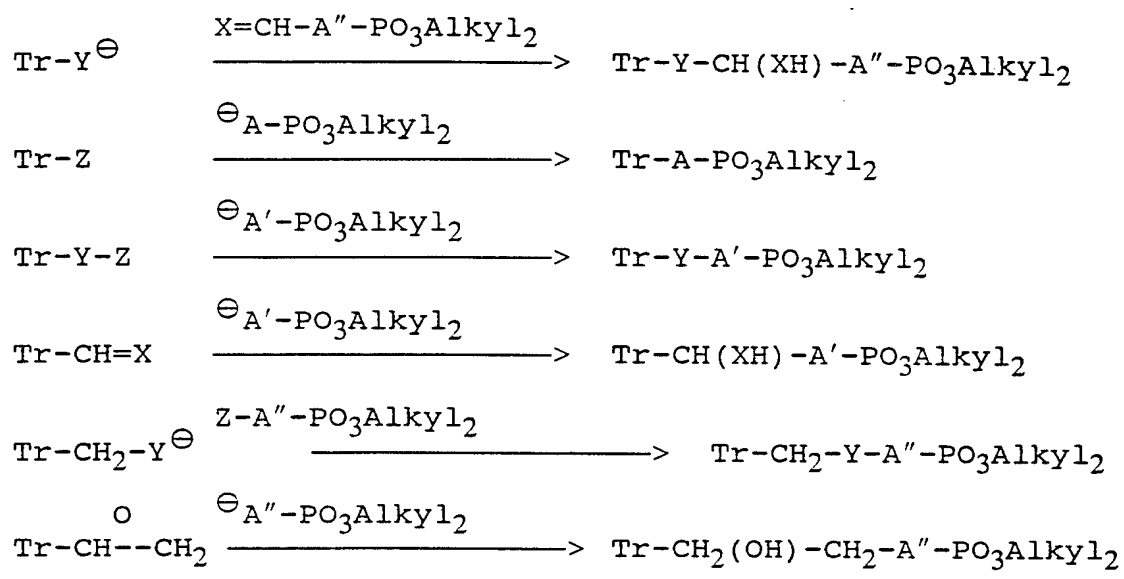
Esters may be prepared for example according to the following reaction schemes.

$$Tr^{\ominus} \xrightarrow{Z\text{-}A\text{-}PO_3Alkyl_2} Tr\text{-}A\text{-}PO_3Alkyl_2$$

$$Tr^{\ominus} \xrightarrow{X{=}CH\text{-}A'\text{-}PO_3Alkyl_2} Tr\text{-}CH(XH)\text{-}A'\text{-}PO_3Alkyl_2$$

$$Tr^{\ominus} \xrightarrow{CH_2\text{--}CH\text{-}A''\text{-}PO_3Alkyl_2} Tr\text{-}CH_2\text{-}CH(OH)\text{-}A''\text{-}PO_3Alkyl_2$$

$$Tr\text{-}Y^{\ominus} \xrightarrow{Z\text{-}A'\text{-}PO_3Alkyl_2} Tr\text{-}Y\text{-}A'\text{-}PO_3Alkyl_2$$



Tr = 1,2,4-triazolyl (or a precursor thereof)
Z = a leaving group
A′ = A minus one atom
A″ = A minus two atoms
X = O, N-alkyl or N-aryl
Y = S, NH or optionally substituted methylene

In cases where the esters contain carbonyl or hydroxy moieties in chain A, these can be subjected to functional group transformations, using known methodology, to yield a variety of different substituents as defined under Q. Such interconversions are extensively documented in the literature, for example by R.C. Larock in "Comprehensive Organic Transformations," VCH Publications, New York, 1989. Various other methods of preparation will be clear from the following Examples, which serve to illustrate the invention. Structures of isolated novel compounds were confirmed by elemental and/or other appropriate analyses. Temperatures are in °C.

Example 1

Potassium carbonate (6.9 g) was added to a solution of diethyl (2-bromoethyl)phosphonate (12.1 g) and 3-mercapto-1,2,4-triazole (5 g) in dry dimethylformamide (50 ml). The mixture was heated to 80° under a nitrogen atmosphere for 8 hours. It was allowed to cool to room temperature, poured into water and extracted with dichloromethane. The extract was dried over magnesium sulfate and evaporated. The residue was purified by silica gel column chromatography to give diethyl [2-(1,2,4-triazol-3-ylthio)ethyl]phosphonate, as an oil.

This compound (2.0 g) was dissolved in dry dichloromethane and trimethylsilyl bromide (7.0 ml) added. The mixture was allowed to stand for 22 hours at room temperature and evaporated under reduced pressure. The residue was dissolved in aqueous acetone (1:9; 25 ml) and stirred at room temperature for 2 hours. The mixture was evaporated to give [2-(1,2,4-triazol-3-ylthio)ethyl]phosphonic acid. This was converted to the dilithium salt by adding aqueous lithium hydroxide until the solution was neutral and then evaporating to dryness. (compound 1)
nmr data: $\delta_H(D_2O$ 1.57(2H,m), 2.97(2H,m), 8.10(1H,s)

Example 2

A solution of monoperoxyphthalic acid magnesium salt hexahydrate (2.10 g) in water (30 ml) was mixed with a solution of diethyl [2-(1,2,4-triazol-3-ylthio)ethyl]-phosphonate, from Example 1, (2 g) in dichloromethane (60 ml). Benzyltriethylammonium chloride (140 mg) was added and the mixture stirred overnight. Aqueous sodium hydrogen carbonate (100 ml) was added until the mixture was pH 9 and the aqueous phase was saturated with sodium chloride and extracted continuously with dichloromethane for 2 days. The extracts were dried and evaporated and the

residue purified by silica gel column chromatography to give diethyl [2-(1,2,4-triazol-3-ylsulfinyl)ethyl]-phosphonate. This was de-esterified as previously described in Example 1, to give [2-(1,2,4-triazol-3-ylsulfinyl)-ethyl]phosphonic acid, as an oil. In a similar manner to that described in Example 1, this was converted to the dilithium salt. (Compound 2)
nmr data: $\delta_H(D_2O)$ 1.93(2H,m), 3.10(2H,m), 8.70(1H,s)

Example 3

3-Amino-1,2,4-triazole (2.33 g) was suspended in a mixture of ethanol (38 ml) and toluene (19 ml) and diethyl (2-oxoethyl)phosphonate (5 g; prepared as described by J M Varlet et al; Tetrahedron letters, 1981, 37, 1377) was added. The mixture was heated under reflux and a nitrogen atmosphere. Condensing solvent was passed through a pressure equalising dropping funnel containing 4Å sieves to remove water. The solvent was collected until the volume of the reaction vessel had reduced to about half. Fresh solvent was added and the drying/collection process was repeated three times and then the mixture was heated under reflux overnight. It was cooled and evaporated to give diethyl [2-(1,2,4-triazol-3-ylimino)ethyl]phosphonate, as a yellow oil.

This product (7.72 g) was dissolved in dry methanol (100 ml) and sodium borohydride (1.05 g) was added portionwise. The mixture was stirred at room temperature for 2½ hours, poured into saturated aqueous ammonium chloride and extracted with ethyl acetate. The extract was dried and evaporated and the residue purified by silica gel column chromatography to give diethyl [2-(1,2,4-triazol-3-ylamino)ethyl]phosphonate, as a gum.

This product (0.73 g) was dissolved in dichloromethane (40

ml) and trimethylsilyl bromide (1.46 ml) was added. The mixture was stirred under nitrogen at room temperature overnight. Methanol (20 ml) was added and the mixture stirred for one hour. Propylene oxide (3.8 ml) was added. The mixture was allowed to stand for 10 minutes and then stirred for 15 minutes. Dry ether (157 ml) was added and the precipitate collected under a stream of nitrogen and dried to give [2-(1,2,4-triazol-3-ylamino)ethyl]-phosphonic acid, as a hygroscopic solid. (compound 3).
nmr data: $\delta_H(D_2O)$ 1.77(2H,m), 3.35(2H,m), 7.85(1H,s)

Example 4

Sodium hydride (0.05 g; 60% oil dispersion) was added to a solution of 1-trityl-1,2,4-triazole-3-methanol (0.37 g; prepared by reduction of 1-trityl-1,2,4-triazole-3-carboxaldehyde) in dry tetrahydrofuran (5 ml) at 0°. After stirring for 15 minutes, diethyl phosphonomethyl-trifluoromethanesulfonate (0.33 g) was added and the temperature allowed to warm to room temperature. After 5 days, the solvent was evaporated and the residue was partitioned between dichloromethane and brine.The aqueous phase was extracted twice more with dichloromethane and the combined organic extracts were dried and evaporated. Column chromatography on silica gel gave diethyl [(1,2,4-triazol-3-ylmethoxy)methyl]phosphonate.

This compound was treated with trimethylsilyl bromide in a similar manner to that described in Example 1, to give [(1,2,4-triazol-3-ylmethoxy)methyl] phosphonic acid. (compound 4)
nmr data: δ_H (D_2O) 3.60(2H,d), 4.76(2H,s), 8.90(1H,s).

Example 5

Sodium metal (0.87 g) was added to dry ethanol (50 ml) at 2° to give a solution of sodium ethoxide. To this was

added diethyl (mercaptomethyl)phosphonate (7 g; prepared as described by G King et al., J Med Chem, 1985, 28 1668) and then after 20 minutes, N-formyl-2-chloroacetamidrazone (5.2 g; prepared as described by I.Y. Anagisawa et al, J. Med. Chem. 1984, 27, 849). The reaction mixture was allowed to warm to room temperature and stirred overnight, and then filtered and evaporated. The residue was dissolved in toluene and heated at reflux for 1 hour with collection of water via a Dean and Stark apparatus. The solvent was evaporated, and the residue was redissolved in dichloromethane and filtered. Column chromatography on silica gel gave diethyl [(1,2,4-triazol-3-ylmethylthio)-methyl]phosphonate.

This compound was treated with trimethylsilyl bromide in a similar manner to that described in Example 1 to give [(1,2,4-triazol-3-ylmethylthio)methyl]phosphonic acid. (compound 5)
nmr data: $\delta_H(D_2O)$ 2.47 (2H,d), 3.86 (2H,s), 8.91 (1H,s).

Example 6 and 7

3-Chloroperbenzoic acid (4.45 g; 50% pure) was added portionwise to a solution of diethyl [(1,2,4-triazol-3-ylmethylthio)methyl]phosphonate (2.28 g) from Example 5, in dichloromethane (40 ml). The mixture was stirred at room temperature overnight, evaporated to dryness and purified by silica gel chromatography to give diethyl [(1,2,4-triazol-3-ylmethylsulfinyl)methyl]phosphonate and diethyl [(1,2,4-triazol-3-ylmethylsulfonyl)methyl]-phosphonate as oils.

Diethyl [(1,2,4-triazol-3-ylmethylsulfinyl)methyl]-phosphonate was treated with trimethylsilyl bromide following the procedure in Example 1 to give [(1,2,3-triazol-3-ylmethylsulfinyl)methylphosphonic acid].

In a similar manner to that described in Example 1, this was converted to the dilithium salt. (Compound 6)
nmr data: δ_H (D_2O) 2.95(1H,t), 3.13(1H,t), 4.15(1H,d), 4.51(1H,d), 8.08(1H,s), 8.23(1H,s).

Diethyl [(1,2,4-triazol-3-ylmethylsulfonyl)methyl]phonate was de-esterified with trimethylsilyl bromide using the procedure described in Example 1 to give [(1,2,4-triazol-3-ylmethylsulfonyl)methyl]phosphonic acid]. (Compound 7)
nmr data: δ_H(D_2O) 3.30(2H,d), 4.54(2H,s), 8.00(1H,s).

Example 8

Diethyl (mercaptomethyl)phosphonate (1 g) and 1-chloromethyl-1,2,4-triazole (0.67 g) were dissolved in ethanol (10 ml) and potassium hydroxide (0,43 g) in water (0.5 ml) was added at room temperature. After 30 minutes the solvents were evaporated and the residue was redissolved in water and extracted with dichloromethane. The organic phase was dried and evaporated to give diethyl [(1,2,4-triazol-1-ylmethylthio)methyl]phosphonate, as an oil.

This compound was treated with trimethylsilyl bromide, in a similar manner to that described in Example 1 to give [(1,2,4-triazol-1-ylmethylsulfonyl)methyl]phosphonic acid as a white solid. In a similar manner to that described in Example 1, this was converted to the dilithium salt. (compound 8)
nmr data: δ_H(D_2O) 2.3(2H,d), 5.17(2H,s), 7.87(1H,s), 8.41(1H,s).

Example 9

Diethyl [(1,2,4-triazol-1-ylmethylthio)methyl]phosphonate (4.0 g) was dissolved in dichloromethane (60 ml) and the solution was cooled to 3°C before solid 3-chloroperbenzoic

acid (5.47 g; 50% pure) was added so that the temperature did not exceed 10°C. The mixture was left at 4° in a refrigerator overnight. The mixture was evaporated and the residue was purified using silica gel chromatography to give diethyl [(1,2,4-triazol-1-ylmethylsulfinyl)]-phosphonate as an oil.

This compound (2.0 g) was de-esterified in a similar manner to that described in Example 1 to give [(1,2,4-triazol-1-ylmethylsulfinyl)]phosphonic acid, as an oil, which was converted, in a similar manner to that described in Example 1, to the dilithium salt. (Compound 9)

nmr data: $\delta_H(D_2O)$ 2.87(1H,t), 3.08(1H,t), 5.40(1H,d), 5.68(1H,d), 7.96(2H,s), 8.42(2H,s).

Example 10

Diethyl [(1,2,4-triazol-1-ylmethylthio)methyl]phosphonate (4.0 g) was dissolved in dichloromethane (200 ml) and 3-chloroperbenzoic acid (11.5 g; 50% pure) was added. The mixture was heated at reflux for 2½ hours and then left overnight at room temperature. The solvent was evaporated and the product was purified using silica gel chromatography to give diethyl [(1,2,4-triazol-1-ylmethylsulfonyl)methyl]phosphonate as an oil.

This compound was de-esterified in a similar manner to that described in Example 1 to give [(1,2,4-triazol-1-ylmethylsulfonyl)methyl]phosphonic acid, as a white solid. (Compound 10)

nmr data: $\delta_H(D_2O)$ 3.6(2H,d), 5.80(2H,s), 8.20(1H,s), 8.90(1H,s)

Example 11

In a similar manner to Example 3, 3-amino-1,2,4-triazole (0.43 g) and diethyl (3-oxopropyl)phosphonate (1.0 g; prepared as described by J. M. Varlet *et al.*, Tetrahedron, 1981, 37, 1377) were reacted together and reduced with sodium borohydride (0.19 g) to give diethyl [2-(1,2,4-triazol-3-ylamino)propyl]phosphonate.

This product (0.5 g) was de-esterified with trimethylsilyl bromide as described in Example 1 to give [2-(1,2,4-triazol-3-ylamino)propyl]phosphonic acid, as a gum. In a similar manner to that described in Example 1, this was converted to the dilithium salt. (compound 11) nmr data: $\delta_H(D_2O)$ 1.3-1.7(4H,m), 4.8(2H,t), 7.55(1H,s).

Example 12

To a solution of diethyl [2-(trimethylsilyloxycarbonyl)-ethyl]phosphonate (1.0 g; prepared as described by J K Thottathil et al, Tetrahedron Letters, 1984, 25, 4741) in dichloromethane (5 ml) containing four drops of dimethylformamide, was added oxalyl chloride (0.38 ml), dropwise, at 0°. The mixture was allowed to warm to room temperature and after 2 hours the solvents were removed under high vacuum to yield the acid chloride. This was redissolved in dichloromethane (5 ml) and 3-amino-1,2,4-triazole (0.29 g), triethylamine (0.59 ml) and 4-dimethylaminopyridine (0.04 g) were added. The mixture was left overnight at room temperature, filtered and evaporated. This material was heated to 200° in an oil bath for 15 minutes and then purified by silica gel column chromatography to give diethyl [2-(1,2,4-triazol-3-ylaminocarbonyl)ethyl]phosphonate as a white solid.

This product was de-esterified with trimethylsilyl bromide as described in Example 1. The crude product was taken up

in water (1 ml) and precipitated by addition of acetone (20 ml) to give pure [2-(1,2,4-triazol-3-ylaminocarbonyl)-ethyl]phosphonic acid, as a colourless solid. (Compound 12).

nmr data: $\delta_H(D_2O)$ 2.05(2H,m), 2.78(2H,m), 8.25(1H,s) .

Example 13

Butyllithium (2.5 m in hexanes; 192 ml) was added slowly, with stirring, over 30 minutes to trimethyl phosphonate (59.6 g) in dry tetrahydrofuran (500 ml) at -78°, under nitrogen. The mixture was stirred for a further 10 minutes at this temperature. Copper(I)iodide (104.4 g) was added and the mixture was stirred for 10 minutes at -78°, then gradually allowed to warm to -30° and stirred at this temperature for 1 hour. A solution of chloroacetyl chloride (61.8 g) in dry ether (240 ml) was added over 30 minutes. The mixture was gradually allowed to warm to room temperature, stirred for 3 hours and quenched with water (250 ml). The mixture was filtered through a pad of kieselguhr. The filtrate was extracted with ethyl acetate, washed with aqueous sodium thiosulfate, then with brine, dried over magnesium sulfate and evaporated under reduced pressure. The resulting brown oil which was distilled under reduced pressure through a small column, collecting the fraction of b.p. 98-106°/0.3 mm, to give dimethyl (3-chloro-2-oxopropyl)phosphonate, as a pale yellow oil.

1,2,4-Triazole-3-thiol (2.73 g) was added to a solution of potassium hydroxide (1.52 g) in ethanol (50 ml) and water (5 ml) with stirring. To this was added in one portion dimethyl (3-chloro-2-oxopropyl)phosphonate (5.40 g). The solution was stirred at room temperature for 90 minutes then evaporated to dryness. The residue was partitioned between saturated aqueous brine and dichloromethane. The aqueous phase was extracted with dichloromethane and the

extracts were combined and evaporated to give a brown oil, which was purified by silica gel chromatography to give dimethyl [2-oxo-3-(1,2,4-triazol-3-ylthio)propyl]-phosphonate, as a viscous oil.

This compound was de-esterified, in a similar manner to that described in Example 1, to give [2-oxo-3-(1,2,4-triazol-3-ylthio)propyl]phosphonic acid as a brown oil. In a similar manner to that described in Example 1, this was converted to the dilithium salt. (Compound 13)

nmr data: $\delta_H(D_2O)$ 3.12(2H,d), 4.19(2H,s), 8.87(1H,s)

Example 14

Sodium borohydride (0.38 g) was added to a stirred solution of dimethyl [2-oxo-3-(1,2,4-triazol-3-ylthio)propyl]phosphonate (4.20 g) in methanol (50 ml). The mixture was stirred for 24 hours at room temperature and solvent removed under reduced pressure. The residue was dissolved in saturated brine and extracted with dichloromethane. The extract was dried over magnesium sulfate and evaporated to give a viscous oil. This was purified by silica gel column chromatography to give dimethyl [2-hydroxy-3-(1,2,4-triazol-3-ylthio)propyl]-phosphonate, as a clear colourless oil.

This compound was de-esterified in a similar manner to that described in Example 1 to give [2-hydroxy-3-(1,2,4-triazol-3-ylthio)propyl]phosphonic acid. In a similar manner to that described in Example 1, this was converted to the dilithium salt. (Compound 14).

nmr data: δH (D_2O) 1.58(2H,m), 3.01(1H,m), 3.30(1H,m), 3.97(1H,m), 8.09(1H,s)

Example 15

Sodium hydride (60% in oil) (2.68 g)) was added to a mixture of 2-(4-chlorobutyl)-1,3-dioxolane (10 ml), diethyl phosphite (8.63 g) and sodium iodide (1.0 g) in dry dimethoxyethane (9 ml) at room temperature. The reaction mixture was heated at reflux for 36 hours, allowed to cool and then poured into saturated aqueous ammonium chloride. The mixture was extracted with dichloromethane and the extracts were dried and evaporated. Purification by silica gel column chromatography gave [diethyl 4-(1,3-dioxolan-2-yl)butyl]-phosphonate as an oil.

This product (9.5 g) was dissolved in tetrahydrofuran (150 ml) and 5% aqueous hydrochloric acid (80 ml) was added. After standing at room temperature for 3 days, the reaction mixture was poured into water and extracted with dichloromethane. The combined extracts were washed with saturated brine, dried and evaporated to give diethyl 5-oxopentylphosphonate as a colourless oil.

A solution of butyllithium (2.5 M in hexanes; 4.4 ml) was instilled into a cold (-78°C) solution of 1-trityl-1,2,4-triazole (3.1 ml) in dry tetrahydrofuran (90 ml). After 1 hour, a solution of diethyl 5-oxopentylphosphonate (2.2 g) in dry tetrahydrofuran (10 ml) was instilled into the reaction mixture. After a further 2 hours, saturated aqueous ammonium chloride was added and the temperature was allowed to rise to room temperature. The reaction mixture was poured into water and extracted with dichloromethane. The combined extracts were washed with saturated brine, dried and evaporated. Purification by silica gel column chromatography gave diethyl [5-hydroxy-5-(1-trityl-1,2,4-triazol-3-yl)pentyl]phosphonate, as a colourless solid (m.p. 151-153°C).

This product was treated with trimethylsilyl bromide as described in Example 1. The crude product was partitioned between water and dichloromethane. The aqueous phase was washed twice more with dichloromethane and then evaporated to give [5-hydroxy-5-(1,2,4-triazol-3-yl)pentyl]-phosphonic acid, as a colourless solid. In a similar manner to that described in Example 1, this was converted to the dilithium salt. (Compound 15).

nmr data: $\delta_H(D_2O)$ 1.24(6H,m), 1.71(2H,m), 4.72(1H,t), 8.07(1H,s)

Example 16

Sodium bicarbonate (1.5 g) was added to a solution of 1-trityl-1,2,4-triazole-3-carboxaldehyde (5.0 g) and N-methylhydroxylamine hydrochloride (1.5 g) in dry dichloromethane (100 ml) at 0°. The reaction mixture was stirred for 2 hours then filtered and evaporated to give N-(1-trityl-1,2,4-triazol-3-ylidene)methylamine N-oxide as a white foam, m.p. 186-190°.

A solution of this compound (0.9 g) and diethyl 3-butenyl-phosphonate (0.6 g) in toluene (50 ml) was heated at reflux for 18 hours. The solvent was evaporated and the product was purified by silica gel column chromatography to give diethyl [2-[2-methyl-3-(1-trityl-1,2,4-triazol-3-yl)isoxazolidin-5-yl]ethyl]phosphonate.

A sample of this product (0.6 g) was dissolved in methanol (120 ml) and water (25 ml) and then boric acid (0.13 g) and W2-Raney nickel (2.5 ml) were added. The reaction mixture was subjected to atmospheric pressure hydrogenation over a 2 hour period. The catalyst was removed by filtration and the solvent was evaporated. The residue was redissolved in ethyl acetate, washed with

water, dried and evaporated to give diethyl [3-hydroxy-5-methylamino-5-(1-trityl-1,2,4-triazol-3-yl)pentyl]-phosphonate.

This product was treated with trimethylsilyl bromide (2 ml) as described in Example 15 to give [3-hydroxy-5-methylamino-5-(1,2,4-triazol-3-yl)pentyl]phosphonic acid as a 3:1 mixture of diastereoisomers. (Compound 16). nmr data: (Where separate peaks clearly occur for different diastereoisomers the chemical shift of the major isomer is underlined): δ_H (D_2O) 1.5 (4H,m), 2.1 (2H,t), 2.4 and 2.5 (3H,2 x s), 3.5 (1H,m), 4.4 and 4.5 (1H,2 x t, 8.50 and 8.55 (1H,2 x s).

Example 17 (FBC 92476)

Sodium hydride (80% in oil) (6.0 g) was added portionwise with ice-bath cooling to a stirred solution of diethyl (hydroxymethyl)phosphonate (33.6 g) in dry ether (300 ml). After complete addition the slurry was stirred at room temperature for 2 hours to form a clear solution. Epichlorohydrin (147 g), and cetyltriethylammonium bromide (0.5 g) were added and the solution was stirred at room temperature for 3 days. After evaporation, the residual liquid was partitioned between dichloromethane and water (approximately 400 ml of each). The organic layer was dried over magnesium sulfate and evaporated to give a liquid residue. This was distilled under reduced pressure to give diethyl (oxiranylmethoxymethyl)phosphonate, b.p. 113-4°/0.45 mm.

Sodium metal (0.55 g) was added to dry ethanol (40 ml) at 10° to give a solution of sodium ethoxide. To this was added a solution of 1,2,4-triazole (1.54 g) in dry ethanol (5 ml). The mixture was stirred for 15 mins and a solution of diethyl (oxiranylmethoxymethyl)phosphonate (5.0 g) in

dry ethanol (10 ml) was added. The mixture was stirred for 16 hours at room temperature and heated under reflux for 1 hour. It was evaporated to dryness and redissolved in saturated aqueous ammonium chloride and subjected to continuous extraction with dichloromethane for 20 hours. The extracts were dried and evaporated and the residue purified by silica gel chromatography to give diethyl [2-hydroxy-(1,2,4-triazol-1-yl)propoxymethyl]phosphonate, as a colourless oil.

A sample of this compound was dissolved in 6M hydrochloric acid and heated under reflux overnight. The solution was evaporated under reduced pressure to give [2-hydroxy-(1,2,4-triazol-1-yl)propoxymethyl]phosphonic acid, as an oil. In a similar manner to that described in Example 1, this was converted to the diammonium salt (Compound 17) nmr data: $\delta_H(D_2O)$ 3.35(1H,m), 3.43(3H,m), 4.02(1H,m), 4.18(2H,m), 7.84(1H,s), 8.25(1H,s)

Example 18

Sodium hydride (60% in oil; 0.29 g) was added to a solution of diethyl (mercaptomethyl)phosphonate (1.15 g) in a 1:1 mixture of tetrahydrofuran and diethyl ether (10 ml). After 10 minutes at room temperature, epichlorohydrin (0.48 ml) was added. The resulting mixture was stirred at room temperature for 45 minutes and then diluted with diethyl ether. The organic phase was separated, washed with water and evaporated to give diethyl (oxiranylmethyl-thiomethyl)phosphonate, as an oil.

A solution of this compound (1.4 g), 1,2,4-triazole (0.42 g) and anhydrous potassium carbonate (0.84 g) in dry 2-butanone (30 ml) was heated under reflux for 7 hours. The mixture was cooled, decanted and evaporated. Following purification by silica gel column chromatography, the

product was de-esterified using 6M hydrochloric acid as described in Example 17 to give [(2-hydroxy-3-(1,2,4-triazol-1-yl)propylthio)methyl]phosphonic acid. In a similar manner to that described in Example 1, this was converted to the dilithium salt. (compound 18)
nmr data: δ_H (D_2O) 2.50(4H,m), 4.03(1H,m), 4.27(2H,m), 7.83(1H,s), 8.30(1H,s).

Example 19

Sodium hydride (60% in oil; 4.8 g) was added portionwise to a solution of diethyl (hydroxymethyl)phosphonate (16.9 g) in dry tetrahydrofuran (200 ml) with cooling by an ice/water bath. The mixture was stirred for 1 hour and then bromoacetaldehyde dimethyl acetal (11.8 ml) was added dropwise over 20 minutes. The mixture was stirred at room temperature for 3 days and then poured into saturated aqueous ammonium chloride and extracted with dichloromethane. The extracts were dried and evaporated, and then purified by silica gel column chromatography to give diethyl [(2,2-dimethoxyethoxy)methyl]phosphonate, as an oil.

This compound (5.9 g) was suspended in 2% aqueous hydrochloric acid and heated at reflux for 10 minutes. The reaction mixture was extracted with dichloromethane and the combined extracts were washed with water, dried and evaporated to give diethyl [(2-oxoethoxy)methyl]-phosphonate, as an oil.

In a similar manner to Example 3, this compound (0.80 g) and 3-amino-1,2,4-triazole (0.32 g) were reacted together and reduced with sodium borohydride (0.8 g) to give diethyl [[2-(1,2,4-triazole-3-ylamino)ethoxy]methyl]-phosphonate.

This product was de-esterified with trimethylsilyl bromide in a similar manner to that detailed in Example 1 to give [[2-(1,2,4-triazol-3-ylamino)ethoxy]methyl]phosphonic acid. In a similar manner to that described in Example 1, this was converted to the diammonium salt. (Compound 19). nmr data: $\delta_H(D_2O)$ 3.28(2H,t), 3.50(2H,d), 3.57(2H,m), 7.69(1H,s).

Example 20

In a similar manner to that described in Example 1, diethyl (2-oxiranylmethoxymethyl)phosphonate (2.3 g) was reacted with 5-mercapto-3-methyl-1,2,4-triazole (1.2 g), followed by deesterification as described in example 17 to give {[2-hydroxy-3-(5-methyl-1,2,4-triazol-3-ylthio)-propoxy]methyl}phosphonate, as an oil. (compound 20) nmr data: $\delta_H(D_2O)$ 2.41(3H,s), 3.07(1H,m), 3.22(1H,m), 3.49(2H,m), 3.53(2H,d), 3.90(1H,m)

Example 21 and 22

1,8-Diazabicyclo[5.4.0]undec-7-ene (0.77 ml), chloromethyl pivaloate (0.88 ml) and sodium iodide (0.15 g) were added sequentially to suspension of trans-[3-hydroxy-3-(1,2,4-triazol-3-yl)cyclohexyl]phosphonic acid, (0.5 g; prepared as described in EP 528760) in dry acetonitrile (10 ml). The mixture was stirred at 70° for 60 hours and evaporated to dryness. The residue was partitioned between brine and ethyl acetate and the aqueous phase extracted twice more with ethyl acetate. The combined organic extracts were dried and evaporated and the residue purified by silica gel column chromatography to give di(pivaloyloxymethyl) trans-[3-hydroxy-3-(1-pivaloyloxy-methyl-1*H*-1,2,4-triazol-5-yl)cyclohexyl]phosphonate, (compound 21), and di(pivaloyloxymethyl) trans-[3-hydroxy-3-(1-pivaloyloxymethyl-1*H*-1,2,4-triazol-3-yl)cyclohexyl]-phosphonate. (compound 22)

nmr data: $\delta_H(CDCl_3)$

compound 21: 1.19(9H,s), 1.25(18H,s), 1.30-2.55(9H,m), 3.59(1H,bs), 5.66(4H,m), 6.26(1H,d), 6.50(1H,d), 7.80(1H,s).

compound 22: 1.19(9H,s), 1.24(18H,s), 1.35-2.60(9H,m), 2.78(1H,bs), 5.67(4H,m), 6.00(2H,s), 8.25(1H,s).

Test Example 1

Fungicide Test - *in vivo*

Compounds are assessed for activity against one or more of the following:

Erysiphe graminis: barley powdery mildew
Leptosphaeria nodorum: glume blotch
Plasmopara viticola: vine downy mildew
Phytophthora infestans: late potato blight
Pyricularia oryzae: rice blast
Venturia inaequalis: apple scab

Aqueous solutions or dispersions of the compounds at the desired concentration, including a wetting agent, were applied by spray or by drenching the stem base of the test plants, as appropriate. Plants or plant parts were then inoculated with appropriate test pathogens and kept under controlled environment conditions suitable for maintaining plant growth and development of the disease. After an appropriate time, the degree of infection of the affected part of the plant was visually estimated. Compounds were considered active if they gave greater than 25% control of the disease at a concentration of 500 ppm (w/v) or less.

The following known compounds were also tested:

Compound A: [3-hydroxy-3-(1,2,4-triazol-3-yl)-cyclohexyl]phosphonic acid, (compound 2.002 in EP 528760).

Activity against the noted pathogens was shown by the following compounds

Erysiphe graminis
A, 17, 21, 22
Leptosphaeria nodorum
A, 11, 18
Phytophthora infestans
A, 21, 22
Plasmopara viticola
A, 4, 6, 13, 17
Pyricularia oryzae
A, 3-6, 18, 21, 22
Venturia inaequalis
7, 15

Test Example 2
Herbicide Test - *in vivo*
a) Pre-Emergence test
In a greenhouse, the noted plant species were treated pre-emergently with the noted compounds of the invention, at the rate shown. The compounds of the invention were sprayed evenly over the vessels containing seeds of the plants as an aqueous acetone solution containing a wetting agent. After 3 to 4 weeks growth, the plants were visually assessed for any herbicidal response.

At least 25% control of the noted weeds, at a rate of 10 kg/ha or less, was shown by the following compounds

Abutilon theophrasti
17
Alopecurus myosuroides
17
Avena fatua
1, 10

Chrysanthemum segetum
17
Echinochloa crus-galli
1, 11, 17
Elymus repens
17
Galium aparine
3, 17,
Pharbitis purpurea
4, 6, 11, 17
Polygonum lapathifolium
11, 17
Setaria viridis
1, 11, 17
Solanum nigrum
3,17

b) Post-emergence test

In a greenhouse, seedlings of the noted plant species were treated post-emergently with the noted compounds of the invention, at the rate shown. The compounds of the invention were sprayed evenly over the vessels containing the plants as an aqueous acetone solution containing a wetting agent. After 3 to 4 weeks growth, the plants were visually assessed for any herbicidal response.

At least 25% control of the noted weeds, at a rate of 10 kg/ha or less, was shown by the following compounds
Abutilon theophrasti
3, 11, 17, 18
Alopecurus myosuroides
1, 11, 17, 18
Avena fatua
2, 11, 17, 18
Chrysanthemum segetum
1, 2, 17, 18

Echinochloa crus-galli
3, 11, 17, 18
Elymus repens
11, 17, 18
Galium aparine
2, 3, 5, 6, 11, 17, 18
Pharbitis purpurea
3, 11, 17, 18
Polygonum lapathifolium
2, 3, 5, 11 17, 18
Setaria viridis
3, 5, 11, 17, 18
Solanum nigrum
1, 5, 11, 17, 18

Test Example 3
Saccharomyces cerevisiae IGPD inhibition assay

B.N. Ames (J. Biol. Chem. 1957, 228, 131) first described an assay method for IGPD which was adapted by T. Kiopotowski and A. Wiater (Arch. Biochem. Biophys. 1965 112, 562) for the determination of yeast IGPD activity. The following IGPD inhibition assay was adapted by us from those described by Kiopotowski and Wiater.

The IGPD assay detects the formation of imidazole acetol phosphate (IAP) from imidazole glycerol phosphate (IGP). The enol form of IAP predominates in strong alkaline solution, and absorbs ultra violet radiation at 290nm.

IGPD from *Saccharomyces cerevisiae* for use in the assay was cloned, overexpressed and isolated as follows:

Mutant oligonucleotides BB3919 (5′ TAAACGAAGGCACATATGACA GAGCAGA 3′) and BB3920 (5′ CATACTGTTCGGATCCTACTTACTGA 3′)

were designed to the open reading frame of the *Saccharomyces cerevisiae* IGPD gene (*HIS*3) from the Gen EMBL data base (Accession Number X03245, Strahl, K. and Davis, R.W. "Promoter mutants of the yeast *HIS*3 gene", J. Mol. Biol. 1981, 152, 553-568). The oligonucleotides were synthesised by British Biotechnology Products Ltd., 4-10 The Quadrant, Barton Lane, Abingdon, Oxon, OX14 37S, UK. BB3939 was designed to include an *Nde*I restriction site (CATATG) at the start codon (ATG) of the *His*3 gene and BB3920 was designed to include a *BamHI*, restriction site (GGATCC) downstream of the *HIS*3 gene stop codon (TGA). *Saccharomyces cerevisiae* (YNN295) genomic DNA was supplied by Cambridge BioScience, 25 Signet Court, Newmarket Road, Cambridge, CB5 8LA, UK.

The *Saccharomyces cerevisiae HIS*3 gene was amplified from genomic DNA using *Taq* DNA polymerise (Boehringer Mannheim UK, (Diagnostics and Biochemicals) Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG, UK). The amplification used 100 ng of *Saccharomyces cerevisiae* DNA; 100 pmoles each of BB3919 and BB3920; 200 mM each of adenosine-5′-triphosphate, cytidine-5′-triphosphate, guanosine-5′-triphosphate and thymidine-5′-triphosphate with 2.5 units of *Taq* DNA polymerase in buffer supplied by the manufacturer. The amplification was performed at 94° for 90 seconds, followed by 25 cycles of 37° for 120 seconds, 72° for 120 seconds and 94° for 60 seconds, finishing with 37° for 120 seconds and 72° for 7 minutes.

The 770 base-pair amplification product was digested with restriction enzymes *Nde*I and *BamHI* and ligated into *Nde*I and *BamHI* - digested pJLA503 to yield pSAL119. The plasmid, pSAL119, was maintained in *Escherichia coli* XL1-BLUE (Stratagene Ltd., Cambridge Innovation Centre, Cambridge Science Park, Milton Road, Cambridge, CB4 4GF, UK. Overexpression of IGPD activity was demonstrated in

pSAL119 using the induction regimes described by Schander, B., Blöchrer, H., Frank, R., McCarthy, J.E.G. in "Inducible Expression Vectors Incorporating the *Escherichia coli atpE* translational initiation region", Gene 1987, 52, 279-283.

Stock cultures of *E. coli* XL-1 Blue transformed with pSAL119 were maintained at 4° on Luria-Bertani (LB) agar V containing ampicillin (50 μg/ml) and tetracycline (20 μg/ml) as described by Maniatis et al, in Molecular Cloning, A Laboratory Manual, published by Cold Spring Harbour, USA, 1982.

Recombinant IGPD for inhibition assays was isolated from pSAL119 transformed *E. coli* XL-1 Blue grown up in liquid culture as follows. LB liquid medium was prepared by dissolving 10 g tryptone, 5 g yeast extract and 5 g sodium chloride per litre of distilled water before autoclaving for 15 minutes. On cooling to room temperature 50 mg ampicillin and 20 mg tetracycline were added to 1 litre of medium. Colonies from stock plates were used to inoculate 100 ml of medium which was incubated with shaking (225 rpm) at 28° until the optical density at 600 nm of the medium had reached 0.5. The medium was then incubated overnight at 42° to induce recombinant protein expression.

Cells were harvested by centrifugation at 8000 x g for 30 minutes at 4°. The cell pellet (1-2 g wet weight) was suspended in 2 ml cold buffer A (30 mM triethanolamine, pH 8.0, also containing 10 mM 2-mercaptoethanol and 50μM manganous chloride) and lysed by sonnication (3 cycles; 30s on; 30s) off at 4°, using a MSE Soniprep 150 (Fisons, Loughborough, UK) .

The soluble fraction was recovered as supernatant after centrifugation of lysate at 15,000 x g for 15 minutes at

4°. The supernatant was removed and buffer exchanged using a prepacked Sephadex G-25 column (PO-10 supplied by Pharmacia Biotech) pre-equilibrated with buffer A. Sephadex G-25 eluate could be stored as 100 µl aliquots at -80°.

"Dilute IGPD solution" was prepared by diluting one volume of Sephadex G-25 eluate with nineteen volumes of buffer A. Using the dilution rate ensured linearity of IGPD activity with respect to time and enzyme concentration, and gives an activity in the IGPD assay of 300-375 mAbs 290nm for 15 minutes incubation period at 37° in the presence of 1.5 mM IGP, (prepared as described by B.N. Ames, J. Biol. Chem. 1957, 228, 131 and B.N. Ames et al, J. Biol. Chem. 1961, 236, 2019).

Samples of compounds to be screened are dissolved in water (or ethanol) to obtain a stock sample solution having a concentration of 10 mM for each compound. "Diluted stock sample solutions" were prepared by diluting one volume with nine volumes of water to obtain a stock sample having a concentration of 1 mM. The dilution was repeated to give a third stock sample solution having a concentration of 0.1 mM.

The assay is conducted using disposable polystyrene test tubes (75 mm x 11 mm). To each tube is added 3 µl of a different stock solution, 275 µl of buffer A and 15 µl dilute IGPD solution. The assay is initiated by adding 10 µl of IGP (15 mm) solution. After this solution is added, the concentration of test compounds is either 100, 10 or 1 µM. The contents of each tube are mixed and all tubes are kept at 37° for 15 minutes. The reaction is stopped by the addition of 600 µl 2 M sodium hydroxide. The contents of each tube are mixed and all tubes are kept at 37° for 30 minutes while the IAP formed during the enzyme reaction

enolises in the now alkaline solution. The absorbence at 290 nm is recorded for every tube with a Shimadzu spectrophotometer (V.A. Howe, Banbury, UK).

The absorbence change due to the conversion of IGP to IAP is then calculated for each sample and the background controls. Compounds that inhibit IGPD reduce the absorbence change.

Percentage activity is calculated using the following formula:

$$\% \text{ activity} = 100 \times \frac{(\Delta A_{\text{Test T15}} - \Delta A_{\text{Test T0}})}{\Delta A_{\text{Control T15}} - \Delta A_{\text{Control T0}})}$$

where ΔA_{Test} is given by tubes containing test compounds,

$\Delta A_{Control}$ is given by tubes containing no test compounds

and T_{15} and T_o refer to the time in minutes when the enzyme assay was stopped.

Percentage inhibition is calculated using the following formula:

$$\% \text{ inhibition} = 100 - \% \text{ activity}$$

At least 20% inhibition at a concentration of 100 μM or less was shown by compounds 4-9, 11, 13-20 and A.

Test Example 4

This example illustrates that *Saccharomyces cerevisiae* and other fungi are inhibited by compounds of the invention and that this inhibition can be reversed by addition of L-histidine.

Methods which are well known to those skilled in the art can be employed to demonstrate that *Saccharomyces cerevisiae* is inhibited by the compounds and that the

inhibition effect is by perturbation of histidine biosynthesis, the biochemical pathway in which imidazole glycerol phosphate dehydratase (IGPD) is involved. *Saccharomyces cerevisiae* wild-type, when grown on minimal medium (yeast nitrogen base, DIFCO, 1.7 g/l; glucose 2%, w/v and agar 1.5%, w/v), is completely inhibited at 500 parts per million (ppm) by Compound A and compound 17. This inhibition is completely reversed by the addition of L-histidine at 20 mg/l, but not with an equivalent concentration of D-histidine.

Similar experiments were conducted and similar results obtained using *Leptosphaeria nodorum* and *Candida albicans*. These data indicate that the fungicidal activity of these compounds is through a perturbation of histidine biosynthesis a pathway in which IGPD is involved.

Test Example 5

This example illustrates that overexpression of IGPD in *Saccharomyces cerevisiae* confers resistance to compounds of the invention.

Those skilled in *in vitro* genetic manipulation will know that overexpression of an inhibitor's target enzyme will often confer resistance to that inhibitor. Such overexpression can be realised, in *Saccharomyces cerevisiae*, by inserting the gene for the target enzyme upstream of a galactose-inducible promoter such as *GAL1* or *GAL10*. Such promoters are present in cloning vectors pYES2.0 (supplied by *Invitrogen* Corporation, San Diego, USA) or pEMBLyex4 (supplied by Dr J Murry, Department of Biotechnology, University of Cambridge).

In this example the wild-type *Saccharomyces cerevisiae* IGPD gene (*HIS3*) was inserted in the "sense" (translatable) orientation and the "antisense"

(untranslatable) orientation with respect to the inducible promoter for both vectors. These manipulations generated four new plasmids: pSAL59 (pYES2.0, with an "antisense" *HIS3* insert); pSAL60 (pYES2.0, with a "sense" *HIS3* insert); pSAL61 (pEMBLyex4, with an "antisense" *HIS3* insert) and pSAL61 (pEMBLyex4 with a "sense" *HIS3* insert).

To confirm that the *HIS3* gene was functional in these constructs, pSAL59-pSAL61 inclusive were transferred to a *Saccharomyces cerevisiae* mutant deficient in IGPD activity (*Saccharomyces cerevisiae* INVSc1, the genotype of which is: *MATα, his3-d1,* (IGPD-deficient); *leu2; trp1-289* and *ura3-52*). *Saccharomyces cerevisiae* INVSc1 normally requires histidine at 20 μg/ml (*his3*), leucine at 60 μg/ml (*leu2*), tryptophan at 40 μg/ml (*trp1*) and uracil at 20 μg/ml (*ura3*) for growth in the minimal medium described above. When carrying copies of either pSAL59, pSAL60, pSAL61 or pSAL62, however, uracil is not required because the *URA3* gene deficient in *Saccharomyces cerevisiae* INVSc1 is carried on the cloning vectors pYES2.0 and pEMBLyex4. Since the *HIS3* gene was cloned into pYES2.0 to generate pSAL59 and pSAL60, and pEMBLyex4 to generate pSAL61 and pSAL62, it was expected that, in case of the "sense" constructs, pSAL60 and pSAL62, these vectors would also allow *Saccharomyces cerevisiae* INVSc1 to grow without histidine in minimal medium. This was demonstrated to be the case. Surprisingly, the "antisense" constructs pSAL59 and pSAL61 also allowed *Saccharomyces cerevisiae* INVSc1 to grow in the absence of histidine in minimal medium. These data, therefore, demonstrate that a functional IGPD gene "*HIS3*" is resident in all four plasmids (pSAL59-pSAL61 inclusive).

Table A demonstrates the elevated resistance to the compounds when *Saccharomyces cerevisiae* INVSc1, containing "sense" constructs pSAL60 and pSAL62, is grown on minimal

medium containing leucine and tryptophan and the expression-inducing carbon source, galactose. Elevated resistance is not manifested when glucose is used as the sole carbon source, a non-inducing carbon source. It is noteworthy that the antisense constructs, in *Saccharomyces cerevisiae* INVSc1, are more sensitive to compound 17 than the wild-type.

TABLE A

RESISTANCE LEVELS (PPM) OF *S CEREVISIAE* STRAINS TO IGPD INHIBITORS: GALACTOSE-INDUCIBLE RESISTANCE IN "SENSE" CONSTRUCTS

		Compound A Carbon Source		Compound 17 Carbon Source	
S. cerevisiae strain	Plasmid	Glucose	Galactose	Glucose	Galactose
wild-type		<500	<500	<500	100-200
INVSc1	pSAL59	<500	<500	<500	<50
INVSc1	pSAL60	<500	>750	<500	>3000
INVSc1	pSAL61	<500	<500	<500	<50
INVSc1	pSAL62	<500	>750	<500	>3000

These genetical data were confirmed at the enzyme level (see Table B) by demonstrating that *Saccharomyces cerevisiae* INVSc1, containing the "sense" constructs pSAL60 and pSAL62 had elevated levels of imidazole glycerol phosphate dehydratase (IGPD) activity. *Saccharomyces cerevisiae* wild-type, INVSc1 and INVSc1

containing pSAL59 to pSAL62 inclusive, were grown in liquid yeast minimal medium described above including the supplements histidine, leucine, tryptophan and uracil at the concentrations described earlier. Raffinose at 4% (w/v) was included as a non-inducible carbon source. After 72 hours incubation, galactose was added to a final concentration of 2% (w/v). After a further 24 hours incubation, the cell cultures were harvested at 13700 x g for 30 minutes at 4°, washed with sterile distilled water and finally re-suspended in 3 ml of cold buffer A described in Test Example 3. The cell suspensions were lysed by sonication and buffer exchanged as described in Text Example A.

Crude enzyme preparations absorb strongly at 290 nm - the detection wavelength used in the IGPD assay. To ensure detection of enzyme activity in these cell-free extracts, a modified assay was used, which determines IGPD activity at 370 nm (after Ames and Mitchell (1955) J. Biol. Chem., 212 687-697). Each of the cell-free extracts was assayed for IGPD activity as described in Test Example 3 with the following alterations. To each tube was added 280 μl of cell-free extracts and the assay was initiated by adding 20 μl of IGP (15 mM) solution. After mixing, the contents of each tube was kept at 30° for 45 minutes. The reaction was stopped by the addition of 30μl 1M perchloric acid. The contents of the tube are mixed and 19μl 1M potassium hydroxide was added to return the solution pH to around pH10. The IAp formed was converted to imidazole acetol (IA) by addition of 2μl alkaline phosphatase (Sigma, UK; 2,500 $U.ml^{-1}$), 4μl 50 mM $MgCl_2$ and 4μl water. After mixing, tubes were incubated at 45° for 20 minutes. This reaction was stopped by the addition of 800 μl 2M sodium hydroxide. IA formed during the alkaline phosphatase reaction quickly enolises in the alkaline solution at room temperature and

the change in absorbance at 370 nm was recorded for every tube between 10 and 15 minutes of the addition of the alkali.

The absorbance change due to the conversion of IGP to IAP to IA is then calculated for each sample and the background controls.

To enable the comparison of the IGPD activities in all 5 cell-free extracts, the protein content of each was determined as follows:

To 20μl of cell-free extracts 1 ml of Coomassie reagent (Pierce) was added, and the absorbance of the solution at 595 nm was measured. A calibration curve was constructed with bovine serum albumin (Sigma, UK) over the concentration range of 75-1000 μg protein/ml.

IGPD activity is calculated using the published extinction coefficient for the enol form of IA (10.4×10^6 nmol/ml/cm; Ames and Mitchell (1955) J. Biol. Chem., 212 68-697).

and the following formula:

$$\text{Activity (nmol/h/mg)} = \frac{100 \times (\Delta A_{\text{Test T45}} - \Delta A_{\text{Test T0}}) \times 60}{(10.4 \times 10^6 \text{ nmol/ml/cm}) \times 45 \times \text{protein content (mg/ml)}}$$

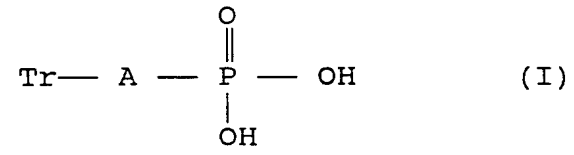
TABLE B

S. cerevisiae strain	plasmid	IGPD activity nmol/h/mg
Wild-type	-	nd
INVSc 1	pSAL59	nd
INVSc 1	pSAL60	80
INVSc 1	pSAL61	nd
INVSc 1	pSAL62	35

These data demonstrate that different levels of gene expression confer different levels of resistance to the compounds, lower levels least resistance, higher levels most resistance. This correlation confirms that the mode of action of these compounds is through the inhibition of IGPD.

CLAIMS

1. The use for combating fungi, of a compound of formula I



(I)

where

Tr is optionally substituted 1,2,4-triazol-1-yl or 1,2,4-triazol-3-yl;

A is

a) Q, where Q is an optionally substituted chain containing 3 to 6 atoms, and in which

(i) when the chain is of 3 atoms, at least one of the chain atoms is a hetero atom,

(ii) when the chain comprises 4 carbon atoms, then the second carbon from the triazole is not substituted by optionally substituted hydroxy, and

(iii) when the chain is of 3 to 5 atoms, the phospho group is not attached directly to an oxygen atom; or

b) an optionally substituted three membered carbon chain or ring, in which two optional substituents can together with the chain atoms form a carbocyclic ring; and in which when A is a chain, then

(i) when the carbon adjacent Tr is substituted by oxo or optionally substituted hydroxy, at least one of the other chain carbons is substituted, and/or two optional substituents can together with the chain atoms form a carbocyclic ring;

(ii) when Tr is unsubstituted 1,2,4-triazol-1-yl and the carbon adjacent Tr is unsubstituted or substituted by alkyl, aryl or haloaryl, the middle chain carbon is not substituted by hydroxy,

together with esters, salts and complexes with metal salts

thereof.

2. Compounds of formula I as defined in claim 1, in which A is as defined under (a) as well as acyloxyalkyl esters of compounds where A is as defined under (b).

3 A pesticidal composition comprising a compound as claimed in claim 2, in admixture with an agriculturally acceptable diluent or carrier.

4 A pharmacuetical composition comprising a compound as claimed in claim 2, in admixture with an pharmacologically acceptable diluent or carrier.

5. A method for identifying potential fungicides which comprised testing a candidate compound in an imidazole glycerol phosphate dehydratase inhibition assay.

6. Fungicides identified by the method claimed in claim 4.

7. The use as a fungicide of a compound which is an imidazole glycerol phosphate dehydratase inhibitor, with the proviso that the compound is not a general enzyme inhibitor and is not a compound previously known to have fungicidal activity.

8. The use according to claim 7 wherein the imidazole glycerol phosphate dehydratase inhibitor is one which produces at least 20% reduction in imidazole glycerol phosphate dehydratase activity when tested against a fungal enzyme preparation at 100 μM.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 94/02568

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01N57/24 A01N61/00 C07F9/6518

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01N C07F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 19629 (JAPAT) 12 November 1992 cited in the application see claim 15 ---	1,6-8
X	GB,A,2 158 071 (ICI) 6 November 1985 cited in the application see claims 7,8 ---	6-8
X	EP,A,0 511 826 (ROHM AND HAAS) 4 November 1992 see page 18, compound 67 ---	2
X	EP,A,0 438 375 (CIBA-GEIGY) 24 July 1991 see claim 1 ---	2
X	EP,A,0 170 228 (BOEHRINGER) 5 February 1986 see claims ---	2,4
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 February 1995	16. 03. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Decorte, D

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 94/02568

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BE,A,644 164 (BAYER) 15 June 1964 see claims 2-4	2,3
X	DE,A,29 24 600 (HOECHST) 22 January 1981 see claims	2,3
A	CHEMICAL ABSTRACTS, vol. 121, no. 9, 29 August 1994, Columbus, Ohio, US; abstract no. 101852b, T.R. HAWKES ET AL. 'Iimidazole glycerol phosphate dehydratase: a herbicide target.' see abstract & BRIGHTON CROP PROT. CONF.--WEEDS, vol.2, 1993 pages 739 - 744	5

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/GB 94/02568

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		US-A-	4687767	18-08-87
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DE-A-2924600	22-01-81	NONE		